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## Epigenetic therapy in myeloproliferative neoplasms: evidence and perspectives

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### Abstract

The classic Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs), which include polycythaemia vera, essential thrombocythaemia and primary myelofibrosis, originate from a stem cell-derived clonal myeloproliferation that manifests itself with variable haematopoietic cell lineage involvement; they are characterized by a high degree of similarities and the chance to transform each to the other and to evolve into acute leukaemia. Their molecular pathogenesis has been associated with recurrent acquired mutations in janus kinase 2 (JAK2) and myeloproliferative leukemia virus oncogene (MPL). These discoveries have simplified the diagnostic approach and provided a number of clues to understanding the phenotypic expression of MPNs; furthermore, they represented a framework for developing and/or testing in clinical trials small molecules acting as tyrosine kinase inhibitors. On the other hand, evidence of abnormal epigenetic gene regulation as a mechanism potentially contributing to the pathogenesis and the phenotypic diversity of MPNs is still scanty; however, study of epigenetics in MPNs represents an active field of research. The first clinical trials with epigenetic drugs have been completed recently, whereas others are still ongoing; results have been variable and at present do not allow any firm conclusion. Novel basic and translational information concerning epigenetic gene regulation in MPNs and the perspectives for therapy will be critically addressed in this review.

**Keywords:** myeloproliferative neoplasms • epigenetics • JAK2 mutation • methylation • histone deacetylases • MPL mutation

### The myeloproliferative neoplasms

According to the 2008 classification system for haematologic malignancies from the World Health Organization (WHO) the category of myeloproliferative neoplasms (MPNs) includes chronic myelogenous leukaemia (CML, which is marked by the t(9;22)(q34;q11) translocation-*BCR/ABL* rearrangement), polycythaemia vera (PV), essential thrombocythaemia (ET), primary myelofibrosis (PMF), mastocytosis, chronic eosinophilic leukaemia not otherwise specified (CEL-NOS), chronic neutrophilic leukaemia (CNL) and 'MPN, unclassifiable' [1]. In this review, we

will focus only on the so-called 'classic' MPNs, that is PV, ET and PMF, with reference to other MPNs if appropriate. These disorders, whose original identification is credited to W. Dameshek in 1951 [2], share a number of common features [3] that include: the origin in a multi-potent haematopoietic stem cell; an expanded pool of mature cells and precursors with preserved cellular maturation; discrete overlap in the clinical phenotype, and the possibility to transform each into the other or to evolve to acute myeloid leukaemia (AML) [4]. A revision [5] to the previous 2001 WHO

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classification has been prompted by discoveries in 2005 of recurrent mutations in janus kinase 2 (JAK2; JAK2V617F) [6–9] or MPL (MPLW515L/K) [10] and of JAK2 exon 12 mutations [11], which have rapidly improved knowledge on pathogenetic aspects of the diseases and simplified the diagnostic approach. Several recent reviews on these issues have been published [12–16].

PV and ET are relatively indolent disorders [17] that result in a modest reduction of survival, particularly evident after the first decade far from diagnosis; on the contrary, PMF has a more severe course with median survival of about 5 years, although younger patients with low-risk disease may experience survival in excess of 10 years. The most clinically relevant events that occur during the course of PV or ET are arterial and venous thrombosis, haemorrhage, evolution to post-polycythemic or post-thrombocythemic myelofibrosis [18] and transformation to AML [19–21]. In the observational arm of the European collaborative study on low-dose aspirin in PV (ECLAP), the largest study available that included 1638 patients, thrombotic events and transformation to myelofibrosis or AML were responsible for 41% and 13% of all fatalities [20, 22]; accordingly, patients with PV or ET are currently stratified according to the risk of cardiovascular events [23]. Age older than 60 years and/or a previous history of thrombosis allow to identify a category of patients with 'high-risk' disease who, unlike the others, are candidate to cytoreductive therapy [20, 24, 25]; in addition, low-dose aspirin is recommended in all PV patients independent of risk category [26] as well as in most patients with ET. The most commonly used cytoreductive agent is hydroxyurea (HU) because of its proven effectiveness in reducing life-threatening cardiovascular events [27–29]. However, the safety of HU as concerns the risk of transformation to acute leukaemia is still an unsettled issue [16, 23], although most evidence are against a significant raised rate of occurrence [22]. On the other hand, the risk of leukaemia was significantly increased in patients who received other chemotherapeutics in combination or in sequence with HU [22, 30], or who were treated with radiophosphorus [22] or chlorambucil [31].

In patients with PMF, the major causes of death are represented by portal hypertension or hepatic/splenoportal thrombosis and their complications, heart failure, infections, pulmonary hypertension, bleeding, thromboses and leukaemia transformation [21, 32]. Patients with low- and high-risk disease with significantly different survival can be identified based on prognostic scoring systems [33, 34]. Stratification according to the risk is particularly relevant for younger patients who can potentially exploit the curative effect of allogeneic haematopoietic stem cell transplantation [35]; in fact, conventional drug therapy does not appreciably modify the course of disease and it is reserved to patients who present anaemia or have symptomatic splenomegaly.

In summary, therapy for MPNs is mainly employed to counteract signs and symptoms of myeloproliferation, to prevent thrombosis and for the treatment of anaemia or thrombocytopenia in myelofibrosis; however, with the possible exception of interferon- $\alpha$  (IFN- $\alpha$ ) in PV according to recent reports [36–38], none of the agents currently employed proved able to significantly affect the abnormally proliferating myeloid clone.

## The molecular pathogenesis of MPN

The first recurrent molecular abnormalities in the classic MPN have been identified only a few years ago. The JAK2V617F mutation, as a result of a G to T nucleotide shift at nt 1849 in exon 14, is located in the tyrosine kinase-like domain-2 (JH2) pseudo-kinase domain of JAK2 and likely results in the loss of auto-inhibitory control and/or cytokine-induced hyper-activation of JAK2 [39]. Expression of V617F-mutated allele in cytokine-dependent cell lines conferred cytokine independence and cytokine hypersensitivity through the constitutive activation of signal transducers and activators of transcription-5 (STAT5), v-akt murine thymoma viral oncogene homolog 1 (AKT) and extracellular signal-regulated kinase (ERK)-dependent pathways [13, 40]; in addition, mice transplanted with marrow cells transduced with a retrovirus expressing JAK2V617F invariably developed erythrocytosis [6, 41–44] eventually associated with leucocytosis, splenomegaly and later changes suggestive of transformation to post-polycythemic myelofibrosis [41–44]. More recently, transgenic mice presenting an expression of mutated allele lower than wild-type one have been generated, and found to develop an ET-like phenotype [45, 46]; overall these data suggest that the JAK2V617F mutation is an integral component of the myeloproliferative process that underlies the different classic MPNs. The frequency of JAK2V617F mutation is estimated at over 95% in PV, 60% in ET or PMF [47, 48] as well as >50% in patients with the infrequent entity 'refractory anaemia with ringed sideroblasts and thrombocytosis' (RARS-T) [49, 50]. The mutation can be found on one or both alleles (homozygosity) as a result of a mitotic recombination process that occurs in most patients with PV or PMF and a minority only of ET [6, 8, 9]. Additional complex mutations, deletions or insertions have been detected in exon 12 of JAK2 and are usually associated with clinical features typical of a JAK2V617F-negative PV or idiopathic erythrocytosis [11, 51]. Finally, somatic mutations involving codon 515 of MPL [10, 52] (the receptor for thrombopoietin, named after myeloproliferative leukaemia virus oncogene homologue) are located in the cytoplasmic juxtamembrane portion; the commonest are represented by W515L (a tryptophan to leucine substitution) and W515K (a tryptophan to lysine substitution). They are detected in  $\approx$ 10% of patients with PMF [10, 52, 53] or  $\approx$ 8% of JAK2V617F-negative ET [54, 55]. MPLW515L introduction in cell lines resulted in a cytokine-independent growth and hyper-sensitivity to thrombopoietin [56], while its expression in a transplant murine model caused a rapidly fatal disease reminiscent of PMF [10]. Interestingly, in some patients multiple MPL mutations or the coexistence with JAK2V617F allele have been reported [53, 55, 57].

There are some hypotheses to explain the association of JAK2V617F mutation with at least three different clinical phenotypes [58, 59]. One is the so-called 'gene-dosage' hypothesis that suggests that disease-associated phenotypes are the result of the relative proportion of mutated and wild-type allele, with ET (the lowest) and PV or post-polycythemic myelofibrosis (the highest) located at the opposite of the spectrum. Second, JAK2V617F may not be the initial clonogenic event in MPNs and a predicted

'pre-JAK2' mutated cell exists as the founder of clonal haematopoiesis that acquires JAK2 mutation as a secondary event [60, 61]. This hypothesis is also supported by recent findings of mutations in ten-eleven translocation-2 (TET-2) reported by the Vainchenker's group [62]. Finally, there can be a role also for inherited host genetic characteristics, such as the single nucleotide polymorphisms (SNPs) in JAK2 that have been associated preferentially with the diagnosis of PV rather than the other MPNs [63], or generic host characteristics (gender, iron availability, hormones). Furthermore, it can be suggested that subtle changes in gene expression because of epigenetic regulation also contribute, in a non-mutually exclusive but rather cooperative manner with the previous events, to the MPN phenotypic variability. After all, it is a matter of speculation if PV, ET and PMF are separate diseases or different presentations/different phases of a unique disease [64].

## Epigenetic events in cancer

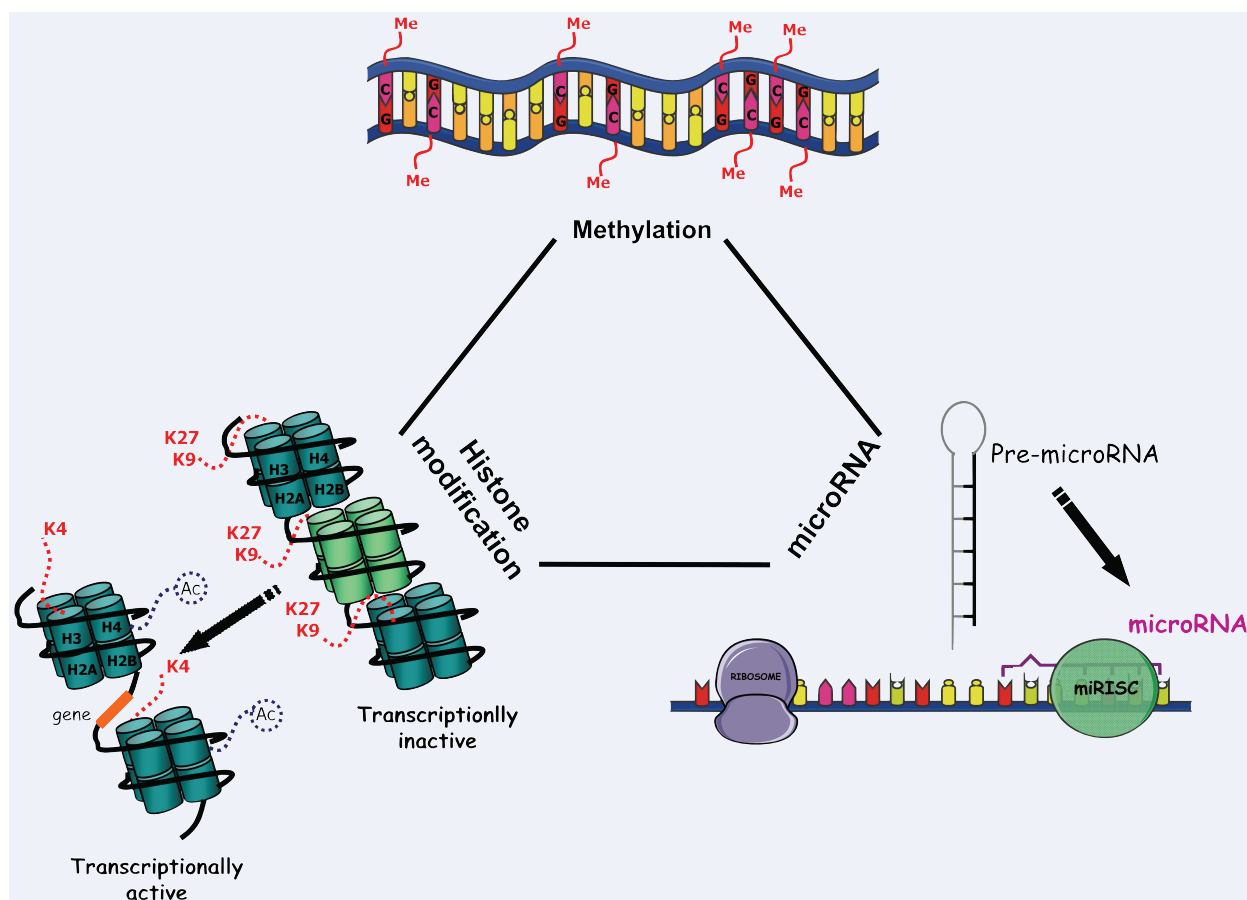
Epigenetic changes are cell-heritable, potentially modifiable abnormalities that affect gene expression [65]. There are two main classic mechanisms that control gene expression through epigenetic changes: DNA methylation and acetylation of histone proteins. However, although most efforts in understanding epigenetics and in developing epigenetic therapy have been directed against DNA methylation and histone acetylation, additional epigenetic alterations are probably at least as relevant as the previous two; these include histone methylation that can be associated either with transcriptional activation or repression [66, 67], or other key molecules involved in the regulation of gene expression, such as micro-ribonucleic acids (microRNAs) [68] (Fig. 1).

DNA methylation occurs when a cytosine residue at a 'CpG' site, that is a cytosine that precedes a guanine, becomes covalently bound with a methyl group at the 5-carbon position, a reaction that is catalyzed by a family of DNA methyltransferases. There are CpG dinucleotide-rich regions in specific locations along the genome that are called 'CpG islands', usually at the 5' regulatory region of many genes; when there is a high degree of methylation in this CpG-rich regions of promoter, the gene cannot be efficiently transcribed. Promoter methylation is a normal process for the orchestrated control of transcriptional activity during development and cell-lineage specification, but it also contributes substantially to tumourigenesis. Hypermethylation at specific gene loci, particularly of tumour-suppressor genes, and global hypomethylation of genomic DNA, as well as inactivation of microRNA genes by DNA methylation [69, 70], are all common findings in human cancer cells [69–71]. Reduced global methylation in cancer cells is mainly because hypomethylation of DNA repetitive sequences, coding regions and introns, particularly in areas of the genome characterized by a low content of coding genes, and increases further during cancer progression [72]. Mitotic recombination and chromosomal rearrangements can be facilitated by global DNA hypomethylation [73], as suggested by the observation that mice

carrying a diffuse genome hypomethylation as a result of a hypomorphic DNA methyltransferase 1 (DNMT1) allele develop aggressive T-cell lymphomas associated with a high rate of chromosome 15 trisomy [74] and by the profound structural alterations of chromosome pericentromeric regions in the rare 'immunodeficiency-centromeric instability-facial anomalies (ICF)' syndrome (OMIM 242860) caused by germ-line mutations in DNMT3b [75]. Loss of methylation in normally silent regions of the genome could also cause the inappropriate expression of genes normally silenced, including imprinted genes or genes of the inactive X chromosome. On the opposite, abnormal gain in DNA methylation with aberrant silencing of transcription may occur at specific gene promoter regions and represents a mechanism for inactivation of tumour-suppressor genes. Examples are the hypermethylation of von Hippel Lindau gene in renal cancers or the cell-cycle control gene p16 in many types of cancer [76]. A tumour type-specific 'hypermethyloma' corresponds to the profile of CpG island hypermethylation in both sporadic cancers and inherited cancer syndromes; in the latter, occurring of abnormal methylation can be considered as the second lesion in a multiple-hit tumourigenesis model [77]. The mechanistic relevance of gene promoter hypermethylation in genes that do not apparently function as tumour-suppressor genes is a matter of discussion, but it is very likely that these might contribute to the phenotypic diversity of cancer; for example, promoter hypermethylation-induced silencing of tissue inhibitor of metalloproteinases-3 (TIMP-3), which encodes for a protease inhibitor, may result in facilitation of tumour cell dissemination [78].

Hypermethylation of CpG islands in gene promoter regions is often associated with specific modification of histones; however, in some instances gene silencing in association with modified histone profile has been observed even in the absence of CpG island hypermethylation. As a matter of fact, differential chemical modifications of histone tail, that is acetylation and methylation, can affect not only DNA replication and DNA repair, but also gene transcription. Acetylation of histones is accomplished by the competing activities of histone acetyl transferase (HAT) and histone deacetylases (HDAC). Acetylated histones have a reduced interaction with the negatively charged DNA, because of lowered positive charge of lysine residue in the presence of acetyl group, and on turn this facilitates the interaction of transcription factors to consensus target sequences on gene promoter [79]. However, although drugs acting as HDAC inhibitors can facilitate the re-expression of genes whose promoter sequence is not hypermethylated, they are generally little or not effective in contrasting gene silencing when gene regulatory sequences are hypermethylated. A sequential treatment scheduling, which involves exposure to a demethylating agent followed by HDAC inhibitors, usually results in synergistic re-expression of silenced genes, at least in most culture conditions [80].

Mature microRNAs are short (19–25 nucleotides) RNAs originated from the cleavage of a 70–100 nucleotide hairpin precursor (pre-microRNA) that hybridize to complementary mRNA targets and either lead to their degradation or prevent their translation of corresponding protein, at least in part depending on the degree of



**Fig. 1** The figure schematizes the main epigenetic mechanisms involved in regulation of gene expression, that is DNA methylation, histone modifications and non-coding small RNAs, the microRNAs. These mechanisms appear to be interconnected on multiple levels. DNA methylation occurs almost exclusively on cytosine preceding guanine pairs in short DNA regions characterized by more than 55% of CG (the 'CpG islands'), and is achieved by the addition of a methyl group to 5' position of a cytosine ring mediated by DNMTs. Most CpG 'islands' are located in gene promoter regions and are normally un-methylated, unlike the sporadic CpG sites in the rest of the genome that are methylated. Methylation of CpG islands in promoter regions is often associated with gene silencing. Histone modifications result in condensed DNA. H3-H4 tetramer and two H2A-H2B dimers form the octamer that constitutes the core histones around which two helical turns of DNA wrap. Acetylation is associated with active gene transcription, whereas other histone modifications correspond to condensed and inactive chromatin. In fact, methylation of H3 at K4 is closely linked to transcriptional activation whereas methylation of H3 at K9 or K27 is associated with transcriptional repression. Whereas classical epigenetics mechanisms regulate gene expression at the transcriptional level, microRNAs function mainly at the post-transcriptional level. The miRNA guides RISC to mRNA target and then miRISC cleaves, degrades or suppress translation of the target mRNA depending on the degree of complementarity between miRNA and mRNA. Me, methyl group; C, cytosine; G, guanine; Ac, acetyl group; Met, methylation; K, lysine; miRISC, RNA-induced silencing complex (RISC) that incorporates the guide strand (miRNA) and the target mRNA.

nucleotide complementarity [81–83]. Expression of microRNAs is tissue and developmental stage specific, and as such they are involved in normal cell homeostasis; however, they are also being increasingly recognized as a new class of genes that are altered in human malignancies and can eventually function as oncogenes (onco-microRNAs) [84]. On the contrary, some microRNAs may have tumour-suppressor function, as argue by their down-regulation in certain tumours; for example let-7 and mir15/mir-16 control the expression of ras sarcoma (RAS) [85] and B cell lymphoma gene-2 (BCL-2) [86], respectively. A role of microRNAs in

haematologic malignancies is supported by recent studies, and they have also been proposed as prognostic markers and therapeutic targets in chronic and acute leukaemias [87]. However, the fine mechanisms that tune the transcriptional expression of microRNAs are still largely unknown, and may depend on the binding of transcription factors to promoter [88, 89] as well as on differential methylation of the microRNA gene or of putative 5' regulatory sequences [90, 91]. In turn, recent evidence suggests that miRNAs could also play important roles in controlling DNA methylation and histone modification [92].

**Table 1** Abnormal gene methylation in MPNs

Gene symbol	Gene nomenclature	Methy* N/Y	Details	Reference
<b>ABL1</b>	V-abl Abelson murine leukaemia viral oncogene homologue-1	Y	Less than 10% of patients	[168]
<b>CALCA</b>	Calcitonin	Y	Mainly in PMF	[169]
<b>p14 ARF</b>	Cyclin-dependent kinase inhibitor 2A, isoform 4	N		[108, 170]
<b>p15</b>	Cyclin-dependent kinase inhibitor 2b			
<b>p16</b>	Cyclin-dependent kinase inhibitor 2a			
<b>Rb</b>	Retinoblastoma-associated protein			
<b>hMLH1</b>	DNA mismatch repair protein Mlh1			
<b>hMSH2</b>	DNA mismatch repair protein Mlh2			
<b>APC</b>	Adenomatous polyposis coli protein			
<b>DAPK1</b>	Death-associated protein kinase 1			
<b>p15</b>	Cyclin-dependent kinase inhibitor 2b	Y	In 20–40% of PMF patients in leukaemic transformation	[171]
<b>p16</b>	Cyclin-dependent kinase inhibitor 2a			
<b>NPM1</b>	Nucleophosmin	N		[172]
<b>CDH1</b>	Epithelial cadherin	N		[108]
<b>p73</b>	p53-like transcription factor			
<b>TIMP-2/-3</b>	Tissue inhibitors of matrix metalloproteinases			
<b>MGMT</b>	O <sup>6</sup> -methylguanine DNA transferase			
<b>RASSF1A</b>	RAS effector homologue 1			
<b>SHP-1</b>	SH2 domain containing tyrosine phosphatase			
<b>RARB</b>	Retinoic acid receptor- $\beta$	Y	Putative oncosuppressor	[173]
		N		[108]
<b>TGF<math>\beta</math>RII</b>	Transforming factor- $\beta$ receptor II	N		[174, 175]
<b>PRV-1</b>	CD177, polycythaemia rubra vera protein 1	Y		[110]
<b>CXCR4</b>	Stromal cell-derived factor 1 receptor	Y		[114, 115]
<b>SOCS1</b>	Suppressor of cytokine signalling 1	N	Variable results possibly because of different regions analysed	[101, 103, 108]
		Y		
<b>SOCS3</b>	Suppressor of cytokine signaling 3	Y	Mainly in PMF. Different promoter regions analyzed in different studies	[101–103]

\*Indicates whether significant hypermethylation of gene promoter was found.

N refers to studies where  $\leq 10\%$  of cases presented evidence of gene promoter hypermethylation.

## Epigenetic events in MPN

Studies on epigenetic gene regulation in MPNs are still scanty and have been focused on single genes credited as being potentially involved in the pathogenesis of these disorders. Table 1 lists published studies that reported an analysis of gene methylation in cells from MPN patients; in most instances, no evidence for abnormal hypermethylation of the genes was found, while some notable exceptions will be discussed briefly here. Preliminary

results of genome-wide methylation analysis pointed to a high rate of gene methylation in PMF patients [93]; furthermore, a unique profile of enhanced histone deacetylase enzyme activity has been described in these patients [94].

Much attention has been devoted to the study of gene methylation status of suppressors of cytokine signalling family proteins (SOCS), particularly SOCS1 and SOCS3. These proteins are rapidly induced following receptor binding of several cytokines, including erythropoietin, interleukin-3 (IL-3) and GM-CSF; in turn, they function as negative regulators of the JAK/STAT pathway by



**Table 2** Compounds acting as epigenetic drugs that are under study for the treatment of human cancer

Drug class	Drug name	Clinical development stage	Diseases	Notes/Company
Histone deacetylase inhibitors				
Short-chain fatty acids				
	Butyrate/phenylbutyrate	Phase I	Refractory solid tumours	
	Valproic acid	Phase I/II	Advanced solid tumours, MDS, refractory AML, CLL	Approved for seizures Abbott Lab. (Abbott Park, IL, USA)
Cyclic tetrapeptides				
	Depsipeptide	Phase I/III	Refractory solid tumours, CLL, AML, CTCL, TCL	Gloucester Pharm. Inc. (Cambridge, MA, USA)
Hydroxamic acids				
	SAHA	Phase I	Refractory solid tumours, NHD, HD, ML, MM, AML, MDS, MPNs, CML, CLL, CTCL, DLBCL, MCL, NK-TCL, GvHD	Approved for CTCL (Vorinostat) Merck & Co (North Wales, PA, USA)
	LBH589/LAQ824	Phase I/II	Refractory solid tumours TCL, CTCL, HD, AML, MDS, CML, ALL, MM	Novartis Corp. (Basel, Switzerland)
	PXD101	Phase I/II	Refractory solid tumours, DLBCL, CLL, MM	Curagen Corp. (Branford, CT, USA), TopoTarget (Copenhagen, Denmark)
	ITF2357	Phase I/II	MPNs, HD, CLL	Italfarmaco (Milan, Italy)
Amides				
	MS-275	Phase I/III	Refractory solid tumours, Lymphomas, AML, MDS, CML	Bayer Schering (Berlin, Germany)
	MGCD0103	Phase II	Advanced solid tumours, NHD	Methylgene Inc. (Quebec, Canada)
	CI-994	Phase II	Advanced solid tumours	Pfizer Inc. (New York, NY, USA)
DNMT inhibitors				
	Azacitidine	Phase I/II	MDS, AML, MM, CML, LLC, NK-TCL, FL, advanced solid tumours	CELGENE Corp. (Summit, NJ, USA)
	Decitabine	Phase I/II/III	MDS, AML, MPNs, CML, ALL, advanced solid tumours	MDS Pharma (Lincoln, NE, USA)

Information derived from the National Cancer Institute web site (<http://www.cancer.gov/search/SearchClinicalTrialsAdvanced.aspx>).

MDS, myelodysplastic syndrome; AML, acute myeloid leukaemia; MPNs, myeloproliferative neoplasms; CML, chronic myelogenous leukaemia; CLL, chronic lymphoid leukaemia; ALL, acute lymphoblastic leukaemia; HD, Hodgkin disease; FL, follicular lymphoma; NHD, non-Hodgkin disease; TCL, T-cell lymphoma; MM, multiple myeloma; DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; NK-TCL, natural killer T-cell lymphoma; CTCL, cutaneous T-cell lymphoma; GvHD, graft *versus* host disease.

reducing the phosphorylation of both JAK and STAT, preventing STAT dimerization and its translocation to the nucleus, and finally resulting in reduced transcription of target genes [95]. Interestingly, the negative regulatory function of SOCS3 may be lost in MPN cells, because SOCS3 was actually found to be promoting, rather than reducing, the proliferation of cells expressing JAK2V617F, possibly through a JAK2V617F-induced enhanced phosphorylation of the protein itself [96]. Previous data indicated that SOCS1 can function as a tumour-suppressor gene and its down-regulation contributes to tumour progression [97]; SOCS1 down-regulation as a result of gene hypermethylation was detected in several neoplasia, including CML [98] where it corre-

lated with poor cytogenetic response to IFN- $\alpha$  [99]. Similarly, hypermethylation of SOCS3 promoter with reduced protein levels was described in several solid tumours. Hypermethylation of SOCS3 in MPNs has been detected in several studies [100–103], particularly in PMF patients [103]; unexplainably, promoter methylation did not correlate with a low level of transcription, although a trend towards an association between SOCS3 methylation and reduced protein was observed in the subgroup of JAK2V617F-negative patients [101]. Of interest, SOCS3 hypermethylation was also detected in six ET females negative for the JAK2V617F or MPLW515L/K mutation who had clonal haematopoiesis [100]. Results concerning SOCS1 methylation

have been more conflicting, and may largely depend on the genomic region employed for methylation status analysis. SOCS1 hypermethylation has been variably reported in AML [104, 105], CML [98, 106] and myelodysplastic syndromes [107]. Conversely, a minority only of MPN patients were reported to have SOCS1 hypermethylation [102, 108].

Abnormally high levels of the polycythaemia rubra vera-1 (PRV-1) gene, encoding for the GPI-linked CD177 protein, are expressed by MPN neutrophils [109], although the levels of mature protein do not differ at all compared to control neutrophils. PRV-1 expression in PV or ET patients has been associated with a reduced level of methylation of CpG sites located close to the transcription start site [110], and decitabine decreased PRV-1 methylation and increased PRV-1 mRNA levels in the KG1 and KG1a leukaemic cell lines. Interestingly, methylation of PRV-1 correlated inversely with the presence of JAK2V617F mutation, and the degree of methylation at CpG 30 (the most conserved site of methylation in PRV-1 up-stream sequence) showed a significant inverse correlation with JAK2V617F allele burden [110].

The constitutive mobilization of haematopoietic stem CD34<sup>+</sup> cells in the peripheral blood is a characteristic finding in patients with PMF [111]; the abnormally high number of circulating CD34<sup>+</sup> cells has been associated with reduced membrane expression of C-X-C chemokine receptor type 4 (CXCR4), which is the receptor for stromal-derived factor-1 (SDF-1), the major chemoattractant for normal haematopoietic stem and progenitor cells [112, 113]. It has been found that CXCR4 is epigenetically regulated and that the extent of methylation of unique CpG islands of the promoter is abnormally high in PMF compared to control cells and is correlated with the protein content on cell membrane [114]. A short-term treatment with 5-Aza reduced promoter methylation, increased membrane expression of CXCR4 and resulted in improved migration of CD34<sup>+</sup> cells in response to SDF-1 *in vitro* [114]. Similar findings have been reported after long-term incubation of PMF CD34<sup>+</sup> cells exposed to 5-Aza followed by trichostatin A [115], and this treatment corrected bone marrow seeding of treated cells once infused in non-obese diabetic severe combined immunodeficiency (NOD/SCID) mice [116]. Importantly, it was shown that this combined treatment also caused reduced proliferation of PMF CD34<sup>+</sup> (as opposite to normal cells) and resulted in significant reduction in the proportion of JAK2V617F mutated cells and of mutated cells harbouring the mutation in an homozygous status, suggesting a preferential effect on cells of the mutated clone [115].

How somatic mutations and abnormal epigenetic gene regulation interact in the pathophysiology of MPNs is largely unknown, but recent data lend support to suggesting a functional link between the increased tyrosine kinase activity of JAK2 and epigenetically regulated genes. Over-activation of the *Hopscotch* gene, a JAK homologue gene in *Drosophila Melanogaster*, led to a global disruption of heterochromatic gene silencing and promoted tumorigenesis, indicating that the JAK/STAT pathway normally regulates cellular epigenetic status [117]. Consistently, binding of STAT3 to promoter and enhancer sequences of the DNA methyltransferase 1 gene (DNMT1) in malignant T-cell lymphoma cells resulted in induction of DNMT1 expression, which in turn supported STAT3 persistent activation

[118]. Overall, these data provided a framework for supporting a direct involvement of aberrant JAK/STAT signalling in epigenetic silencing of genes important for cellular transformation.

Finally, unique expression profiles of microRNAs in granulocytes [119] or other mature haematopoietic cell sub-populations [120, 121] from MPN patients have been characterized; a general down-regulation of microRNAs was found, as reported in other cancer cells, with some notable exception of microRNAs that were overexpressed, some of them in a JAK2V617F-associated manner [119, 120]. However, information about validated gene targets and the mechanisms underlying differential expression of microRNA in MPNs is still lacking [59].

## Drugs for epigenetic therapy

Drugs acting on epigenetic control of gene expression fall into two broad categories: inhibitors of methyltransferases and histone deacetylase (HDAC) inhibitors (listed in Table 2). Among them, those most widely employed are azacitidine, decitabine and vorinostat. These drugs have been used in phase I trials in patients with haematological and solid cancers, and several phase II trials are underway (Table 2). Among myeloid malignancies, most experience has been collected in patients with myelodysplastic syndromes and AML [122].

Hypomethylating agents such as azacitidine and decitabine act by inhibiting DNMT1 and cause DNA hypomethylation leading to induction of silenced genes involved in cellular differentiation and proliferation. Although the demethylating effect depends on incorporation of the drug into DNA, the cytotoxicity of azacitidine is additionally as a result of halting of protein synthesis because the drug is incorporated into RNA as well. Their clinical efficacy in haematological malignancies has been demonstrated *in vitro* and in a series of phase I and II trials. Azacitidine was first approved by FDA in 2004 for the treatment of myelodysplastic syndrome (MDS), based on two phase II [cancer and leukemia group B (CALGB) 8421 and 8921] and one phase III (CALGB 9221) trials in which azacitidine at 75 mg/m<sup>2</sup>/day for 7 days every 28 days used by continuous intravenous infusion or subcutaneous administration was compared with the best supportive care. The phase II trials recorded complete remission (CR), partial remission (PR) and haematological improvement (HI) rates of 15%, 2% and 27%, and of 17%, 0% and 23% in the CALGB 8421 and CALGB 8921, respectively [123]. A subsequent phase III randomized trial in 191 MDS patients reported an overall response rate of 60% on the azacitidine arm (CR, 7%) compared with 5% of patients receiving supportive care; median time to leukaemic transformation or death was significantly prolonged by azacitidine (21 *versus* 13 months). A recent re-analysis of the three CALGB trials by applying WHO classification and International Working Group (IWG) response criteria confirmed those response figures, with 90% of patients achieving a response by six cycles; however, whereas quality of life significantly ameliorated, there was no



improvement in overall survival in the whole patient population or in the separate classes of risk [124]. The combination of azacitidine with histone deacetylase inhibitors, such as sodium phenylbutyrate [125], valproic acid and all-trans retinoic acid [126], has been explored with little evidence of improvement in patients with leukaemia or high-risk MDS.

Decitabine was approved by FDA in the 2006 for the treatment of MDS on the basis of a multi-centre trial of 170 patients with intermediate-2 and high-risk international prognostic score system (IPSS) disease, randomized *versus* best supportive care [127]; overall response rate was 17% (CR 9%, PR 8%) with 13% of patients achieving HI. The median time to response was 3 months with a response duration of 10.3 months, although there was no difference between the two arms in time to AML or death. Significant activity at low, very well-tolerated, doses was reported [128]. Evidence of partially demethylated epigenotypes, including re-establishment of normal p15 protein expression early after drug administration, suggested pharmacologic demethylation as the therapeutic mechanism of action. Decitabine has been used also in imatinib-resistant CML [129] or in combination with imatinib in patients with accelerated or leukaemic-phase CML [130].

Vorinostat, suberoylanilide hydroxamic acid (SAHA), is an inhibitor of class I and II HDAC enzymes, and promotes cell-cycle arrest and apoptosis of cancer cells [131]; relevant target genes have been characterized through gene expression analysis [132]. Vorinostat has received FDA approval for the treatment of progressive, persistent or recurrent on, or after, two systemic therapies T-cell cutaneous lymphoma [133]. In a phase I/II study, vorinostat was used to treat 41 patients with leukaemia or MDS who were relapsed or refractory to previous therapy or who were not candidate to chemotherapy [134]. Haematologic improvement was observed in 17% of cases including two complete responses in AML. Evidence of histone H3 acetylation was found in peripheral blood and bone marrow cells, and down-regulation of proliferation-associated genes was associated with haematologic improvement [134]. A phase IIa trial with vorinostat in patients with lower-risk myelodysplastic syndromes (NCT00486720) and a phase I/II trial of vorinostat in combination with azacitidine (NCT00392353) are currently underway; preliminary results from phase I of the combination trial indicated that the therapy is safe and well tolerated and appears superior to azacitidine alone for time to response, overall response and CR rate [135].

The HDAC inhibitory activity of valproic acid (VPA), an anti-epileptic drug, is known since 2001; VPA induces differentiation and apoptosis in malignant myeloid cell lines. Clinical activity has been demonstrated in studies in MDS patients who received VPA orally on a continuous schedule to maintain a serum concentration of 50–100 µg/ml. The first pilot study reported a 44% overall response rate in MDS with a median response duration of 4 months [136]. In a follow-up study on 122 patients with MSD and AML an overall response rate of 20% was reported, including one CR; a higher percentage of response was observed in low-risk MDS, according to morphological subtype [137]. VPA

has been used in combination with all-trans retinoic acid in patients with acute leukaemia, eventually in association with cytotoxic therapy, without appreciable or with only minor improvements [138–140].

ITF2357 is a new synthetic HDAC class I inhibitor that possess a potent anti-proliferative and pro-apoptotic activity against cells of AML and multiple myeloma [141]. An intriguing property of HDAC inhibitors relies on their ability to down-modulate several soluble cytokines secreted by blood cells as well as from accessory cells of the bone marrow microenvironment [142, 143]. The autocrine and paracrine secretion of different cytokines may play an important role for the neoplastic proliferation of myeloid precursor cells, and possibly represents a relevant target of new anti-neoplastic drugs. Most strikingly, ITF2357 inhibits production of IL-6, vascular endothelial growth factor (VEGF) and IFN-γ, with an IC<sub>50</sub> similar to that required for apoptosis induction of leukaemic cells (0.25–0.5 µM). Despite its *in vitro* and *in vivo* anti-tumour activity, ITF2357 showed little toxicity against normal cells such as mesenchymal stem cells, hepatocytes and peripheral blood mononuclear cells (MNCs), and thrombocytopenia and gastrointestinal toxicity represent the most common side effects after its use in normal volunteers and patients [144]. Of note, similarly to other HDAC inhibitors, ITF2357 induces a significant up-regulation of the cell cycle inhibitor p21.

Because it is predicted that over 30% of human genes are regulated by microRNAs [145, 146], and miRNAs can function either as oncosuppressors or oncogenes [147], it is easily anticipated that miRNAs and anti-miRNA drugs have great potential to be developed as a novel class of epigenetically active drugs for the treatment of cancer. Specific knockdown of miRNAs by anti-miRNA oligonucleotides ('antagomirs') has been pursued *in vitro* and *in vivo*. Krutzfeldt and coworkers showed for the first time the long-lasting and non-toxic silencing generated by intravenously injected antagomir in mice [148]. Inhibition of miR-122 resulted in a reduction of cholesterol levels and a decrease in hepatic fatty acids and cholesterol synthesis in normal mice and in diet-induced obese mice. Several recent papers have generated intriguing data concerning the systemic [149, 150] or intra-tumoural injection [151–153] of antagomiRs, for example in the treatment of melanoma, neuroblastoma or prostate cancer xenografts, where sizeable reduction of tumour growth could be ascertained. To the best of our knowledge, there is no experience in MPNs or other haematologic disorders.

## Epigenetic therapy in MPN

Only a few trials using epigenetically active drugs have been completed or are ongoing in the classic MPNs. 5-azacitidine has been used in patients with refractory/relapsed PMF and post-polycythemic/post-thrombocythemic MF in two phase II trials that differed in the drug scheduling. In the first [154], 34 patients received 5-Aza at 75 mg/m<sup>2</sup> daily for 7 days every 4 weeks

subcutaneously (total cycle dose, 525 mg/m<sup>2</sup>), while in the second trial, a 5-day weekly schedule (total dose, 375 mg/m<sup>2</sup>) was employed in 10 patients [155]. Responses were classified according to the International Working Group for Myelofibrosis Research and Treatment (IWG-MRT) criteria [156]. No patient among those receiving the reduced dosage schedule achieved even a clinical improvement, the lowest response according to IWG-MRT criteria; however, only two of the 10 patients had received more than three of the six planned cycles of therapy because of toxicity, disease progression or patient choice. Of note, 75% to 90% of responses in MDS have been observed between cycles 4 and 6 of therapy, respectively [124], not dissimilar from the median time of 5 months that was recorded in the higher-dose trial in MF [154]. In this study, one patient presented a partial response (3%) and clinical improvement occurred in 21% of the patients; the partial response was maintained for +22 months whereas median duration of clinical improvement was 4 months. Neutropenia was the main adverse effect with grade 3–4 severity in 29% of the patients, and thrombocytopenia all grades occurred in 9%; grade 3–4 non-neutropenic infections were reported in 26% of the patients. A kinetic analysis of global methylation of genomic DNA, measured according to a long interspersed nucleotide element (LINE) bisulphite pyrosequencing [157], demonstrated a gradual and significant decline of LINE methylation. Although changes in the extent of methylation were not significantly different between responders and non-responders, the former presented a trend towards a more rapid hypomethylating effect.

Preliminary results from a phase II multi-centre study with decitabine in 21 patients with PMF have been reported [158]. Seven patients (37%) responded, including two with blast phase-MF; there was one CR, two PR and four HI. Median time to response was two cycles and median duration of response was 5 months, with two patients maintaining their responses at +2 and +14 months, respectively. There was a 61% reduction in the count of circulating CD34<sup>+</sup> cells in that distinguished responders from non-responders.

The safety and efficacy of the HDAC inhibitor ITF2357 in the treatment of patients with PV, ET or MF have been evaluated in a phase II study. The rationale of the clinical trial derived from results on the *in vitro* activity of ITF2357 in cells from PV and ET patients carrying the JAK2V617F mutation. In these experiments, it was shown that: (i) cells carrying the mutated JAK2 are sensitive in colony assays to a 100- to 500-fold lower dose of ITF2357 as compared to cells bearing wild-type JAK2; (ii) in a classical endogenous erythroid colony (EEC) assay, ITF2357 promotes the out-growth of normal colonies over that of JAK2V617F mutated cells; (iii) ITF2357 induces down-modulation of JAK2V617F but not JAK2 wild-type protein in human erythroleukemia (HEL) cells, and (iv) that JAK2V617F inhibition takes place at the post-transcriptional level [159]. ITF2357 was evaluated in PV, ET or MF patients refractory to hydroxyurea or in younger patients who were in need of cytoreductive therapy [160]. The drug was given orally at a starting daily dose of 50 mg b.i.d. that could be escalated to 50 mg t.i.d., in the absence of toxicity. A total of

26 patients (men/women, 12/14), with a median age of 56 (range 36–70) and a confirmed diagnosis of JAK2V617F-positive PV ( $n = 12$ ), ET ( $n = 1$ ), PMF ( $n = 4$ ) or post-polycythemic/post-thrombocythemic MF ( $n = 9$ ) were enrolled in this study. In patients with PV and ET, ITF2357 was well tolerated when given up to 50 mg t.i.d. (no Grade 4 toxicity) and the haematologic response (CR+PR) was 50% at 12 weeks and 40% at 24 weeks. A rapid and sustained improvement of pruritus was observed in 80% and a reduction of splenomegaly in 50% of patients. In MF patients, the haematologic response (major, moderate and minor) was 33% and a partial response of splenomegaly was seen in 23% [160]. Based on this promising activity of ITF2357, different drug dosing and drug combinations are now under investigation.

There is a single report of vorinostat in a patient with a JAK2V617F-positive post-essential thrombocythaemia myelofibrosis who showed improvement in transfusion-dependent anaemia and thrombocytopenia [161]. Modest improvement of anaemia and reduction of transfusional support was also serendipitously observed in a patient with PMF who had received valproic acid for epilepsy prophylaxis [162].

## Conclusions

In summary, although preliminary and sometimes conflicting, results of available studies support abnormal epigenetic gene regulation in MPN cells, particularly in PMF [163], as a contributing mechanism to the pathophysiology of these disorders, and maintain that epigenetics could represent a clinically relevant therapeutic target [163]. Results of the first trials with demethylating agents have been dismal, while substantial hope that HDAC inhibitors can be more effective is supported by the preliminary results of ITF2357 trial. It is also conceivable that combining epigenetic therapy with other drugs might be worthwhile and more efficacious. Gene silencing because of hypermethylation is involved in low response and resistance to drugs in solid cancers [164], and re-activation of silenced gene following decitabine was shown able to overcome resistance both *in vitro* and in preclinical models [165, 166]; results of a recent dose-finding trial of decitabine with carboplatin in solid tumours hold much clinical promise. Therefore, one could imagine the opportunity to associate epigenetic therapy either with cytotoxic drugs (hydroxyurea) or with the novel, specific JAK2 inhibitors [163, 167]. The coming years will surely witness great interest and efforts in developing effective, targeted therapy against the long-neglected MPNs.

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